The Influence of Ectotrophic Mycorrhizal Fungi on the Resistance of Pine Roots to Pathogenic Infections. I. Antagonism of Mycorrhizal Fungi to Root Pathogenic Fungi and Soil Bacteria

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ABSTRACT

Antagonism of ectotrophic mycorrhizal fungi to *Phytophthora cinnamomi,* other root pathogenic fungi, and soil bacteria was examined. In agar plate tests, *Laccaria laccata, Lactarius deliciosus, Leucopaxillus cerealis* var. *piceina, Pisolithus tinctorius,* and *Suillus luteus* inhibited growth of nearly half of 48 different fungal root pathogens. *Leucopaxillus cerealis* var. *piceina* inhibited 92% of the test pathogens. Differences in sensitivity of several isolates of *P. cinnamomi* to inhibition by this symbiont were not found. Culture filtrates of *L. cerealis* var. *piceina* were inhibitory also to growth of *P. cinnamomi* and soil bacteria. Zoospore germination was inhibited completely in filtrates of this symbiont. Maximum antibiotic production occurred during the rapid growth phase in liquid culture. Length of culture incubation and temperature strongly influenced production of inhibitory substances by *L. cerealis* var. *piceina* in liquid culture. It grew best from 10 to 20 C, whereas *P. tinctorius* grew best from 30 to 35 C in liquid culture. Phytopathology 59:153-163.

Ectotrophic mycorrhizae are essential for the establishment of tree seedlings, and for their good growth and development in soils low in nutrients. The inability of tree seedlings free from mycorrhizal fungi to grow in soils with low nutrients, and their successful establishment following inoculation of the soil with suitable mycorrhizal fungi, is well documented by reports from various parts of the world (8).

Several theories concerning the benefit afforded trees by ectotrophic mycorrhizae have been presented. Hatch (10) suggested that the increase of root-absorbing surface in direct contact with soil might well explain the more rapid growth of tree seedlings with mycorrhizae. Others have asserted that mycorrhizae aid plants through selective ion absorption and accumulation. Isotopes of phosphorus (17), nitrogen (13), calcium (14), sodium (15), and potassium (9) are absorbed and accumulated more rapidly in mycorrhizal than in nonmycorrhizal roots. Some have reported that mycorrhizal fungi aid their host by absorbing nutrient substances from very slightly soluble soil minerals, organic substances, and forest litter which are not readily available to nonmycorrhizal roots (4, 19, 20).

Zak (23) suggested that ectotrophic mycorrhizal roots may be less susceptible than nonmycorrhizal roots to infection by root pathogens. He postulated that mycorrhizal fungi may protect absorbing roots of trees by: (i) utilizing root carbohydrates and other chemicals, thereby reducing the attractiveness of the root to pathogens; (ii) providing a mechanical barrier to the pathogen in the form of the fungus mantle; (iii) secreting antibiotics which may inhibit or kill potential pathogens; and (iv) attracting, while in mycorrhizal association with the host root, a protective rhizosphere population of other microorganisms.

Mycorrhizal fungi may afford protection also by stimulating host root cells to elaborate inhibitors that may maintain the symbiotic state, and that also serve to inhibit infection by pathogens.

Tubers of several species of *Orchis* and other genera produce orchinol, coumarin, and an unidentified phenolic compound in response to symbiotic parasitism by *Rhizoctonia repens* (5, 6). Orchinol is produced also by *Orchis* tubers in response to infection by several other species of *Rhizoctonia,* and by other endotrophic mycorrhizal and pathogenic fungi. Orchinol was not found in uninfected orchid tubers. The inhibitory spectrum of orchinol includes many fungi and also certain bacteria. Orchinol and the other inhibitory compounds may be active agents in the defense mechanism of the orchid, keeping the fungal symbiont in a balanced symbiotic state. Another consequence of tuber synthesis of these inhibitory compounds is that they protect the tissue not only against reinfection by the fungal symbiont but also against infection by pathogenic organisms.

Many ectotrophic mycorrhizal fungi produce antibiotics in pure culture effective against many root-pathogenic fungi and various bacteria (23). However, these antibiotics have not been demonstrated to be functional as biological control mechanisms in mycorrhizal associations.

This report deals with the antagonism of mycorrhizal fungi to *Phytophthora cinnamomi,* other fungal root pathogens, and soil bacteria. *Phytophthora cinnamomi,* the primary cause of littleleaf disease of shortleaf (*Pinus echinata*) and loblolly (*P. taeda*) pines in the Southeast, was selected as the major test pathogen because it: (i) infects the same meristematic root tissue infected by mycorrhizal fungi; (ii) initiates infection by motile zoospores which are reportedly attracted chemotactically to roots, thus allowing for tests

on the chemical attractiveness of mycorrhizae; and (iii) infects shortleaf and loblolly pines, which are also hosts for several known mycorrhizal fungi.

MATERIALS AND METHODS.—The isolate of *Phytophthora cinnamomi* used for the major part of this research was obtained in 1963 from soil under shortleaf pine with littleleaf symptoms. The species of *Fomes, Polyporus,* and *Sclerotium* were furnished by C. S. Hodges, Jr., Forestry Sciences Laboratory, Research Triangle Park, N.C., and a species of *Armillaria, Cylindrocladium, Fusarium,* and *Poria,* by B. Zak, Forestry Sciences Laboratory, Corvallis, Oregon. The various *Thanatephorus* and *Rhizoctonia* species were furnished by R. T. Sherwood; *Pythium aphanidermatum, P. carolinianum, P. debaryanum, P. paroecandrum, P. periplocum, P. polytylum, P. spinosum,* and *P. ultimum* by J. A. Spencer; the different isolates of *P. cinnamomi* and various other species of *Phytophthora* by the late F. A. Haasis, all of the Department of Plant Pathology, North Carolina State University at Raleigh, N.C. M. E. Gallegly, Department of Plant Pathology, University of West Virginia, Morgantown, West Virginia, furnished the remaining 16 species of *Pythium.*

The standard sources of vegetative inocula were obtained by growing the pathogens for 15 to 20 days at 23 C in darkness in petri plates containing 30 ml of standard V-8 juice agar medium. Sterile cork borers were used to remove 8-mm-diameter inoculum discs containing mycelium (mycelial discs) from the periphery of the culture.

Isolates of the various ectotrophic mycorrhizal fungi were obtained from B. Zak. The species used in this investigation were *Amanita muscaria* (L. ex Fr.) Pers. ex Hooker, *Laccaria laccata* (Scop. ex Fr.) Berk. & Br. [syn. *Clitocybe laccata* (Scop.) Quél.], *Lactarius deliciosus* (L. ex Fr.) S. F. Gray, *Leucopaxillus cerealis* var. *piceina* (Peck) ined. [syn. *C. piceina* Peck, *L. albissimus* (Pk.) Sing. var. *piceinus* (Peck) Singer & Smith and *L. cerealis* (Lasch) Sing.], *Pisolithus tinctorius* (Pers.) Coker & Couch, and *Suillus luteus* [syn. *Boletus luteus* (Linn.) Fr.]. These binomials, according to R. Singer (*personal communication*), are the currently accepted names of these fungi (21). These fungi were reported as mycorrhizal symbionts on roots of aseptic shortleaf pine seedlings by Bryan and Zak (1, 2). They also reported *A. muscaria, L. laccata,* and *S. luteus* as symbionts on roots of aseptic loblolly pine seedlings.

Standard mycorrhizal fungus inocula, in the form of 8-mm-diameter mycelial discs, were removed from 20 to 30-day-old cultures grown at 23 C in darkness in petri plates each containing 30 ml of Hagem agar medium (16). An enriched Melin-Norkrans' (18) nutrient solution (MN nutrient solution) was used for growing the mycorrhizal fungi. This modified medium, referred to hereafter as MMN medium, afforded comparable nutritional conditions for the mycorrhizal fungi in liquid and agar culture tests and in aseptic mycorrhizal synthesis test. The formulations of MN nutrient solution and the MMN medium are as follows:

Ingredient	MN nutrient solution	MMN medium
$CaCl_2$	0.05 g	0.05 g
NaCl	0.025 g	0.025 g
KH_2PO_4	0.5 g	0.5 g
$(NH_4)_2HPO_4$	0.25 g	0.25 g
$MgSO_4 \cdot 7H_2O$	0.15 g	0.15 g
$FeCl_3$ (1%)	1.2 ml	1.2 ml
Thiamine HCl	25 µg	100 µg
Malt extract (paste)		3 g
Sucrose	2.5 g	10 g
Bacto-agar (optional)		15 g
Distilled water	To 1,000 ml	To 1,000 ml

After autoclaving for 15 min at 121 C, the reaction of both the agar and liquid formulations was pH 5.5-5.7.

Agar plate antagonism studies.—Antagonism between mycorrhizal fungi and *P. cinnamomi* was studied by seeding the mycorrhizal fungi 1 cm from the edge of petri plates containing 30 ml of MMN, malt-extract (MEA), potato-dextrose (PDA), or Norkrans' (18) casein hydrolysate agar media (NCH). The mycorrhizal fungi were incubated at 23 C in darkness for 20 days and radial growth recorded.

Phytophthora cinnamomi was introduced at various distances from the mycelial margin of the symbiont, either as zoospores introduced into 1-cm-diameter wells removed by a sterile cork borer, or as 1-cm-diameter mycelial discs placed on the agar surface. A minimum of five replicates was used for each treatment. After incubation at 23 C for 5 days in darkness, zones of inhibition were measured (Fig. 1).

Inocula of nonsterile zoospores of *P. cinnamomi* were used throughout this investigation. Mycelium was grown for 30 to 35 days at 23 C in darkness on V-8 juice:oatmeal agar medium in petri plates. Mycelial felts were stripped free from the agar surface and floated in 25 ml of nonsterile soil leachate in petri plates for 7 days at 23 C. After microscopic examination to confirm sporangial production, the mycelial felts were removed and washed gently with tap water (21 to 24 C) over a Büchner funnel. After 10-15 min, each felt was placed in a 50-ml beaker with 30 ml distilled water, chilled at 5 C for 15 min, and then incubated at room temperature (24 to 26 C) for 1 hr for release of zoospores from sporangia (12). A standard concentration of nonsterile zoospores of 26,000 to 30,000/ml was obtained consistently by this procedure.

Several isolates of *P. cinnamomi* were tested for antagonism by *L. cerealis* var. *piceina* and *P. tinctorius* on MMN agar, MEA, and PDA media. Measurements of the zone of inhibition were made after 5 days' incubation at 23 C.

Various other fungal root pathogens were tested on MMN agar medium for antagonism by the mycorrhizal fungi. This was an attempt to determine the relative biological inhibitory spectrum of each symbiont, and

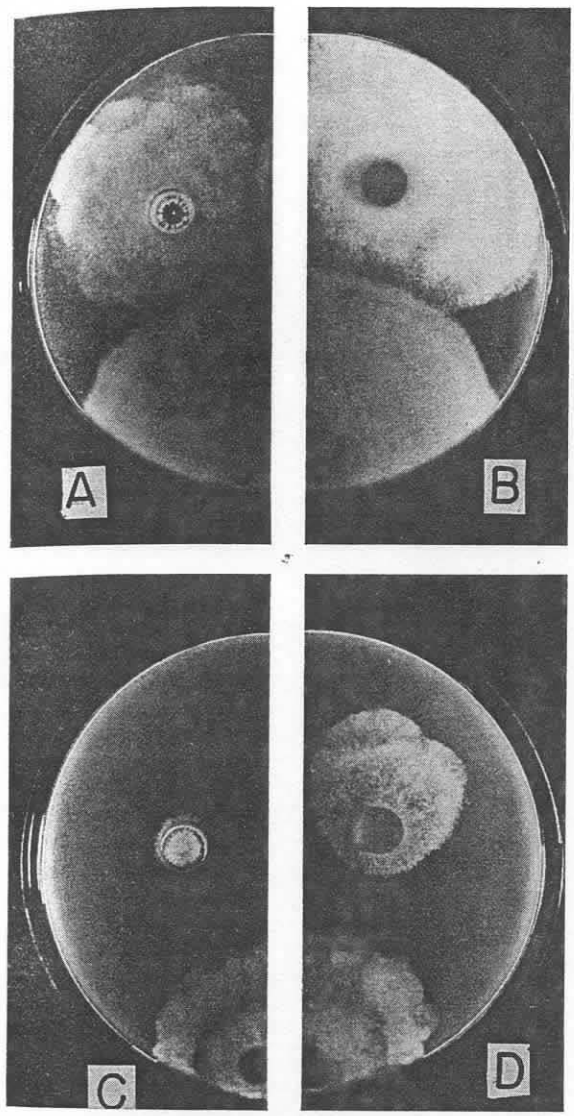

Fig. 1. Agar culture antagonism test. A and B, growth of *Phytophthora cinnamomi* (top fungus) from zoospore **A)** and mycelial disc **B)** inocula toward a noninhibiting mycorrhizal fungus. C and D, zones of growth inhibition from zoospore **C)** and mycelial disc **D)** inocula of *P. cinnamomi* caused by an inhibiting mycorrhizal fungus.

the relative sensitivity of the root pathogens to antagonism by the symbionts. Incubation, as above, was terminated, and zones of inhibition of each pathogen were measured when radial growth on MMN agar reached 4 cm. This 4-cm radial growth period was predetermined for each of these pathogens in the absence of any other fungus.

Liquid culture antagonism studies.—Liquid culture tests were made in 100 ml of MMN or Hagem liquid media in 250-ml Erlenmeyer flasks. All flasks were autoclaved for 15 min at 121 C, with a minimum of three replicates for each treatment. Flasks were seeded with the test fungi and shaken vigorously for 30 sec, and re-randomized in the incubators every 5 days. After incubation the mycelium was harvested on Whatman No. 1 filter paper in a Büchner funnel with slight vacuum applied to facilitate filtering. The filter paper with mycelium was oven-dried at 70 C for 48 hr, and weighed to the nearest 0.5 mg. The oven-dried mycelial weight was obtained by subtracting, from that of the filter paper-mycelial weight, the predetermined oven-dried tare weights of the filter paper, fungal inoculum, and residues retained on filter paper of control-unseeded liquid cultures.

Twenty-five ml of the combined culture filtrate were used to determine pH and the amount of 1 N NaOH required to adjust to pH 6.0. All pH determinations were made electrometrically. The remaining culture filtrate, approximately 220 to 240 ml, was filter-sterilized through HA (0.45 μ pore size) Millipore filters under vacuum and stored in sterile flasks at 5 C in darkness for a maximum of 24 hr. All culture filtrates were aseptically adjusted to pH 6.0 for use in bioassays, except where otherwise stated.

The following bioassay techniques were used throughout this investigation to detect inhibitory substances produced by symbiotic fungi:

(i) *Culture filtrate-agar diffusion bioassay.*—The culture filtrates, before pH adjustment, were pipetted into 150 × 25 mm test tubes in volumes of 40, 20, and 10 ml sterile distilled water was added to the latter two volumes to 40 ml that furnished final dilutions of 1:0, 1:1, and 1:3, respectively. Agar diffusion plates were prepared from 100 × 25 mm plastic petri plates containing 60 ml of MMN agar medium by removing a 2-cm-diameter agar plug from the center of each plate. A zoospore inoculation well, 1-cm-diameter, was made 2 cm from the center well. Two and one-half ml of each test dilution were pipetted into diffusion wells of each of three replicate plates. After incubation at 23 C for 24 hr, this volume was removed by suction and each well recharged with 2.5 ml of the same test dilution. Twenty-four hr later, 0.5 ml of zoospore inoculum of *P. cinnamomi* was introduced into the inoculation well, and a 1-cm-diameter mycelial disc of *P. cinnamomi* was placed on the opposite side of, and 2 cm distant from, the diffusion well. After incubation for 5 days at 23 C in the dark, zones of inhibition around the diffusion wells were measured.

(ii) *Growth of Phytophthora cinnamomi in culture filtrate bioassay.*—Volumes of 15, 5, and 3 ml of culture filtrate were introduced into 150 × 25 mm test tubes. Control liquid medium was added to the latter two volumes to 15 ml. Final dilutions of 1:0, 1:2, and 1:4 were obtained, respectively. Each test dilution was replicated three times. The tubes were seeded with mycelial discs of *P. cinnamomi,* and incubated at 23 C for 7 days in darkness. The oven-dried mycelial weights were determined as previously described. Mycelial discs lacking observable growth after incubation were placed on V-8 juice agar medium to determine whether *P. cinnamomi* was still viable.

(iii) *Zoospore bioassay.*—Zoospore assay plates were prepared by attaching six 18-mm-diameter × 10-mm-deep Pyrex glass rings (2-mm-wall thickness) to the

bottoms of 95 × 22 mm heavy-walled petri plates with RTV-11, a nontoxic silicone product that vulcanizes at room temperature (General Electric Corporation, Silicone Products Division, Waterford, New York). One, 0.5, or 0.25 ml of the culture filtrate or control medium was pipetted into randomly selected rings. Sterile water was added to the latter two volumes to 1 ml. Each dilution was replicated 3 times. One-half ml of zoospore inoculum was pipetted into each ring, resulting in final dilutions of culture filtrate of 2:1, 1:2, and 1:4, respectively. Beginning after approximately 10 min and continuing at 15- to 20-min intervals, the zoospores were examined for motility under 100× magnification with green light illumination. Between examinations the plates were maintained in darkness, because white light inhibited zoospore motility. Quantitative results were obtained by counting the zoospores in several microscopic fields in each replicate ring. After all zoospores in control liquids had encysted, percentage of germination of zoospores was determined in the culture filtrate solutions.

(iv) *Bacterial bioassay.*—Nonsterile soil leachate containing at least 5 distinct types of bacteria was centrifuged at 2,000 rpm for 30 min at 5 C to remove most of the debris and protozoa from suspension. The centrifuged leachate, stored at 5 C for 16 months, was used for all bacterial assays in this investigation. This storage did not noticeably change the types or amount of bacteria originally present. One ml of the soil leachate was incubated without replication with 2 ml of culture filtrate or control liquid medium in 100 × 15 mm test tubes. Nonincubated soil leachate was diluted similarly to determine the number of bacteria present in the inoculum. After 48 hr at 23 C, each tube was shaken vigorously for 5 min, and dilutions with sterile water of 2×10^2, 2×10^4, and 5×10^4 were prepared without replication. Following 30 min of vigorous shaking, 0.1 ml was removed immediately from each test dilution and pipetted onto, and spread evenly over, the surface of each of 3 petri plates containing 30 ml of half strength AC Agar Medium (Difco). Counts of bacterial colonies were made after 48 hr of incubation at 30 C in darkness.

RESULTS.—*Agar medium antagonism studies.*—Most of the mycorrhizal fungi grew best in MMN agar medium, with the exception of *P. tinctorius* and *S. luteus*, which grew equally well in all media (Table 1). *Leucopaxillus cerealis* var. *piceina* strongly inhibited growth of *P. cinnamomi* in all agar media. The other mycorrhizal fungi were either weakly inhibitory on certain media or noninhibitory. Growth of *P. cinnamomi* from zoospores was inhibited consistently more than growth from mycelial discs by *L. cerealis* var. *piceina* and the other symbionts. This was probably due to the great sensitivity of promycelium growing from germinated spores to antibiotics, as suggested by Landy et al. (11).

The inhibition of *P. cinnamomi* by *L. cerealis* var. *piceina* on the agar media was not due to the acidity of the culture, because more acid reactions were recorded from cultures of noninhibitory symbionts. For example, the reading at the mycelial margin of *P. tinctorius* grown in Hagem medium was pH 3.8, with reactions of pH 4.2, 4.6, and 5.2 at 1-, 2-, and 3-cm distances, respectively, from the margin. *Leucopaxillus cerealis* var. *piceina* gave an average reaction of pH 4.1 at the mycelial margin, and 4.4, 4.6, and 4.8 at 1-, 2-, and 3-cm distances, respectively, from the margin. All of these values, as well as those recorded from cultures of the other mycorrhizal fungi, were well within the reaction limits generally considered favorable for growth of *P. cinnamomi* (3). The inhibition of *P. cinnamomi*, therefore, was assumed to be due to an antibiotic(s) produced by *L. cerealis* var. *piceina* and the other mycorrhizal fungi.

Much larger zones of inhibition of *P. cinnamomi* were obtained from *L. cerealis* var. *piceina* cultures grown in Hagem than in MMN medium, even though vegetative growth was greater in the latter. This unequal response was related to gel hardness rather than to quantity of antibiotic produced. The softer Hagem medium had a more rapid diffusion of the antibiotic, as determined by tests with different percentages of agar incorporated into both media.

TABLE 1. Radial growth of pine mycorrhizal fungi after 20 days at 23 C on agar media, and resulting antagonistic effects on the growth of *Phytophthora cinnamomi* from zoospore and mycelial disc inocula

Mycorrhizal fungus	Agar medium[a]	Radial growth (mm)	Inhibition zone (mm) from indicated inoculum	
			Zoospore	Mycelial disc
Amanita muscaria	MMN	6.2	0	0
	Hagem	2.1	0	0
	MEA	1.8	0	0
	PDA	2.2	0	0
	NCH	4.8	0	0
Laccaria laccata	MMN	25.6	0.8	0
	Hagem	17.8	0	0
	MEA	14.4	1.5	1.3
	PDA	19.5	0	0
	NCH	16.6	1.7	1.1
Lactarius deliciosus	MMN	13.6	1.1	0.7
	Hagem	6.2	0	0
	MEA	2.4	0	0
	PDA	0		
	NCH	0		
Leucopaxillus cerealis var. *piceina*	MMN	26.3	24.2	15.4
	Hagem	22.7	28.7	21.4
	MEA	20.8	17.2	12.4
	PDA	18.6	7.4	6.1
	NCH	15.1	16.8	14.2
Pisolithus tinctorius	MMN	19.7	0	0
	Hagem	20.6	0	0
	MEA	20.1	0	0
	PDA	18.4	0	0
	NCH	20.7	0	0
Suillus luteus	MMN	9.7	1.9	1.3
	Hagem	10.1	0	0
	MEA	9.4	0	0
	PDA	10.8	0	0
	NCH	9.6	1.3	0.8

[a] Symbols indicate the following agar media: MMN = modified Melin-Norkrans; MEA = malt-extract; PDA = potato-dextrose; and NCH = Norkrans' casein hydrolysate.

Motility and germination of zoospores of *P. cinnamomi* were inhibited strongly; i.e., zoospores encysted after 15 to 30 min without subsequent germination when incubated in wells within the mycelial mat, at the mycelial margin, or at 0.5 cm from the margin of *L. cerealis* var. *piceina* grown on MMN and Hagem media. Zoospores similarly incubated in cultures of the other mycorrhizal fungi were not inhibited; i.e., zoospores were motile for 6 to 8 hr with approximately 90 to 100% germination.

The antibiotic(s) elaborated by *L. cerealis* var. *piceina* in these tests was antibacterial. The bacteria in the zoospore inoculum did not multiply, whereas bacteria grown with the other fungal symbionts under the same conditions multiplied rapidly in the incubation wells. The antibacterial activity was apparent, especially in wells within the mycelial mat of *L. cerealis* var. *piceina* on either medium. Antibacterial activity was demonstrated also by using the standard streak method in which nonsterile soil leachate was the source of bacterial inoculum. Again, pH per se did not influence inhibition of either zoospores of *P. cinnamomi* or the bacteria, since the mat reactions of *L. cerealis* var. *piceina* on MMN and Hagem media were pH 5.3 and 4.8, comparable to those of the other mycorrhizal fungi with reactions between pH 5.2 and 3.8.

Inhibition of selected fungal root pathogens by five mycorrhizal fungi was evident in 44% (101 of 231) of all antagonism tests (Table 2). The inhibitory spectrum of each symbiont, based on the percentage of pathogens inhibited, was distinct. *Pisolithus tinctorius* did not inhibit any pathogen, whereas *L. deliciosus*, *L. laccata*, and *S. luteus* inhibited approximately 16, 35, and 76% of the pathogens, respectively. The greatest inhibitory spectrum was that of *L. cerealis* var. *piceina*, which inhibited 92% of the test pathogens. The inhibition spectrum of each symbiont, and the differential sensitivity of the pathogens, suggest that as a group these mycorrhizal fungi produced a variety of different antifungal compounds. For example, *L. laccata* only weakly inhibited 1 of the 9 *Phytophthora* spp., but was antagonistic to 12 (50%) of the *Pythium* spp. *Suillus luteus* inhibited all the *Phytophthora* spp. to some extent, and all but 2 of the 24 *Pythium* spp. *Lactarius deliciosus* was antagonistic to all *Phytophthora* spp., as was *S. luteus*, but did not inhibit any *Pythium* spp. *Leucopaxillus cerealis* var. *piceina* inhibited in varying degrees all the root pathogens except *Armillaria mellea*, *Fusarium oxysporum*, *Thanatephorus cucumeris*, and *Rhizoctonia crocorum*.

The most resistant groups to antibiotics produced by the mycorrhizal fungi were species of *Thanatephorus* and *Rhizoctonia*, of which only about 10% were sensitive. The higher Basidiomycetes, *Armillaria*, *Fomes*, *Polyporus*, and *Poria*, were moderately sensitive in that 20% were inhibited. Phycomycetes were the most sensitive group, with 50% of the *Phytophthora* and *Pythium* spp. showing inhibition to varying degrees.

Each isolate of *P. cinnamomi*, regardless of source, geographic origin, or mating type, was inhibited similarly by *L. cerealis* var. *piceina* on the various agar media (Table 3). *Pisolithus tinctorius* did not inhibit any isolate of the pathogen. In fact, mycelia of these isolates overgrew the mycelial margin of this symbiont for 5 to 8 mm in all plates. These results suggest that significant differential sensitivity to the antibiotic(s) produced by *L. cerealis* var. *piceina* does not exist among these isolates of *P. cinnamomi*.

Liquid culture antagonism studies.—All six mycorrhizal fungi grew better in MMN liquid medium than Hagem (Table 4). The reaction of the culture filtrates after 40 days' growth at 25 C was pH 3.4 to 5.0, except for those of *L. deliciosus* and *S. luteus* from MMN medium, which were pH 6.0.

The results of the different bioassays indicated strong antifungal and antibacterial activity in culture filtrates of *L. cerealis* var. *piceina*. Undiluted filtrate of *L. cerealis* var. *piceina* strongly inhibited the growth of *P. cinnamomi* from zoospore and mycelial disc inocula on the agar-diffusion plates. This was more pronounced from MMN medium than from Hagem. The filtrates of the other mycorrhizal fungi from either medium did not inhibit *P. cinnamomi*. Similar results were obtained when *P. cinnamomi* was grown directly in the various culture filtrates. Filtrates of *L. cerealis* var. *piceina* strongly inhibited growth, whereas the filtrates of the other symbionts from either medium did not.

Of the three methods employed, the zoospore bioassay was the most sensitive assay for antifungal activity in filtrates of *L. cerealis* var. *piceina*. The nondiluted MMN filtrate immobilized zoospores in approximately 20 min, and completely inhibited germination. Hagem medium filtrate of the control and of all symbionts except *L. deliciosus* also immobilized zoospores in 20 min. Either *L. deliciosus* removed the factor from Hagem medium, causing rapid loss of motility of zoospores, or produced something which counteracted its effect. Germination of zoospores in excess of 90% occurred in Hagem filtrates of the control and of all symbionts except for those in filtrates of *L. cerealis* var. *piceina*. Germination of zoospores incubated in Hagem filtrates of the latter fungus was inhibited to a lesser degree than those incubated in the MMN filtrates. Inhibition of approximately 40 and 70%, respectively, of zoospore germination was detected also in the 1:2 and 1:4 (filtrate:water) dilutions of the MMN filtrate of *L. cerealis* var. *piceina*. Hagem filtrate dilution of 1:2 inhibited only 30% of the zoospores, and the 1:4 dilution had no effect.

Leachate bacteria multiplied rapidly during incubation in all but *L. cerealis* var. *piceina* filtrates. Bacteria did not multiply in the MMN filtrate of *L. cerealis* var. *piceina;* the number detected after incubation, 0.083 million of cells/ml, was the same number present in the original soil leachate inoculum. Strong bacteriostatic activity was detected also in the Hagem filtrate of this fungus.

In another study, culture filtrates and aqueous mycelial extracts of the aforementioned mycorrhizal fungi from MMN and Hagem liquid media were sterilized by Millipore filtration or by autoclaving for 15 min at 121 C. Bioassays showed negative antibiotic activity in

TABLE 2. Inhibition of various fungal root pathogens by mycorrhizal fungi on modified Melin-Norkrans agar medium

Test pathogen	Mycorrhizal fungus					Pathogen Incubation (days)
	Laccaria laccata	*Lactarius deliciosus*	*Leucopaxillus cerealis* var. *piceina*	*Pisolithus tinctorius*	*Suillus luteus*	
Armillaria mellea Vahl ex Fr.	N[a]	N	N	N	N	20
Cylindrocladium scoparium Morg.	N	N	W	N	M	4
Fomes annosus (Fr.) Cke.	N	N	M	N	N	6
Fusarium oxysporum f. *pini* (Hartig) Snyd. & Hans.	N	N	N	N	M	5
Phytophthora cactorum (Lebert & Cohn) Schroeter	N	W	VS	N	W	6
P. cambivora (Petri) Buisman	N	W	S	N	S	4
P. cinnamomi Rands	N	W	VS	N	W	4
P. citrophthora (Smith & Smith) Leonian	N	NT	VS	N	NT	4
P. crytogea Pethy. & Laff.	N	N	VS	N	W	5
P. drechsleri Tucker	N	NT	VS	N	NT	4
P. heveae Thompson	W	NT	S	N	NT	4
P. palmivora (Butler) Butler	N	W	S	N	S	4
P. parasitica Dastur	N	W	VS	N	S	4
Polyporus tomentosus var. *circinatus* (Fr.) Sact. & Maire	N	W	M	N	N	6
Poria weirii (Murr.) Murr.	N	N	M	N	W	5
Pythium aphanidermatum (Edson) Fitz.	W	N	M	N	S	3
P. aristosporum Vanderpool	M	N	VS	N	S	3
P. carolinianum Matt.	N	N	S	N	W	3
P. debaryanum Heese	M	N	M	N	S	3
P. deliense Meurs.	N	N	M	N	M	3
P. graminicolum Subra	N	N	VS	N	N	3
P. helicoides Drechs.	N	N	S	N	N	3
P. irregulare Buisman	M	N	M	N	S	3
P. mamillatum Meurs.	W	N	M	N	S	3
P. oligandrum Drechs.	W	N	VS	N	VS	3
P. paroecandrum Drechs.	W	N	M	N	S	3
P. periplocum Drechs.	M	N	VS	N	S	3
P. polymorphon Sideris	M	N	VS	N	S	3
P. polytylum Drechs.	N	N	VS	N	W	3
P. proliferans deBary	N	N	S	N	S	3
P. rostratum Butler	M	N	S	N	S	3
P. spinosum Sarvada	W	N	M	N	S	3
P. splendens Braun	N	N	M	N	S	3
P. sp.	M	N	M	N	S	3
P. sp. "A"	M	N	M	N	S	3
P. sp. "B"	M	N	M	N	S	3
P. ultimum Trow	N	N	S	N	M	3
P. undulatum Petersen	M	N	M	N	S	3
P. vexans deBary	W	N	M	N	W	3
Rhizoctonia crocorum (Pers.) D.C.	N	NT	N	N	N	10
R. fuligenea Sappa & Mosca	N	N	NT	N	M	10
R. melongenae Matz	N	N	M	N	W	3
R. mucoroides Bernard	N	N	S	N	N	5
R. repens Bernard	N	W	S	N	N	10
Sclerotium bataticola Taub.	W	N	W	N	N	4
Thanatephorus cucumeris (Frank) Donk	N	N	N	N	N	3
T. cucumeris (*Corticium microsclerotia* Weber)	N	N	W	N	N	4
T. cucumeris [*C. sasakii* (Shirai) Matsumoto]	N	N	W	N	N	3

[a] Inhibition rating symbols indicate the following: VS = very strong, no growth from mycelial disc of test pathogen; S = strong, 14-19 mm zone of inhibition; M = moderate, 6-14 mm zone of inhibition; W = weak, 1-5 mm inhibition zone; N = no inhibition; and NT = not tested.

TABLE 3. Host source, geographic origin, and mating type of isolates of *Phytophthora cinnamomi* and inhibition (mm) caused by *Leucopaxillus cerealis* var. *piceina* on various agar media

Host source	Isolate no.[a]	Geographic origin	Mating type	Inhibition rating[b]		
				MMN[c]	MEA	PDA
Abies fraseri (Pursh) Poir.	100	North Carolina	+	VS	M	W
Camellia japanica L.	352	Georgia	−	VS	S	W
C. japanica	2	Georgia	−	VS	VS	W
Castanea sp.	264	Spain	+	VS	S	M
Castanea sp.	266	France	+	VS	S	M
Chamaecyparis lawsoniana (A. Murr.) Poil.	196	Louisiana	+	VS	S	M
Cinnamomum burmanni Blume	C-275	Sumatra	+	VS	VS	W
Cornus florida L.	158	North Carolina	+	VS	S	W
Macadamia integrifolia Maiden & Betche	166	Hawaii	−	VS	S	W
Persea americana Mill.	334	New Zealand	+	VS	S	W
P. americana	P-128	Peru	+	VS	S	M
Pinus echinata Mill.[d]	281	Georgia	+	VS	S	W
P. radiata D. Don	533	Argentina	+	VS	S	W
Pseudotsuga menziesii (Mirb.) Franco	67	Oregon	+	VS	VS	M
Quercus rubra L.	536	Portugal	+	VS	M	W
Rhododendron obtusum (Lindl.) Planch.	1	North Carolina	+	VS	S	W

[a] Isolate numbers from the late Dr. F. A. Haasis' *Phytophthora* collection.

[b] Inhibition rating symbols indicate the following: VS = very strong, no growth from mycelial disc of *P. cinnamomi*; S = strong, 14-19 mm zone of inhibition with most growth from mycelial disc away from mycelial margin of *L. cerealis* var. *piceina*; M = moderate, 6-14 mm zone of inhibition; W = weak, 1-5 mm zone of inhibition.

[c] Symbols indicate the following agar media: MMN = modified Melin-Norkrans' nutrient; MEA = malt-extract; PDA = potato-dextrose.

[d] Shortleaf pine isolate used predominantly in this investigation and included in Haasis' collection.

mycelial extracts of any symbiont. Zoospore and bacterial inhibition was detected in filter-sterilized filtrates of *L. cerealis* var. *piceina* similar to that reported previously. Autoclaving the filtrates of *L. cerealis* var. *piceina*, however, reduced by half the antifungal and antibacterial activity, indicating the antibiotic(s) is heat-labile.

Antibiotic activity of culture filtrates of L. cerealis var. piceina and P. tinctorius as influenced by time and temperature of incubation.—Preliminary studies indicated that duration of culture incubation strongly influenced the antibiotic activity of cultures of *Leucopaxillus cerealis* var. *piceina*. This study was designed to study the influence of temperature and length of culture incubations on antibiotic production by this symbiont.

Leucopaxillus cerealis var. *piceina* and *Pisolithus tinctorius* were grown in MMN liquid medium for 3 days at 25 C to confirm growth from mycelial disc inoculations. Flasks of the two fungi and control medium were then incubated at 10, 15, 20, 25, 30, 35, and 40 C (± 1 C). After 20 days, mycelium and culture filtrates were harvested from 3 flasks of each fungus and control from each temperature. This was repeated, thereafter, at 10-day intervals for a total of 70 days' incubation. All filtrates were tested for biological activity by the various bioassays.

Leucopaxillus cerealis var. *piceina* grew best at temperatures between 10 and 25 C with poor growth at 30 C (Fig. 2). Growth did not occur at 35 or 40 C, but the fungus was still viable after 70 days' incubation at these temperatures. Autolysis of the mycelium of *L. cerealis* var. *piceina* was apparent after 60 days' incubation at all temperatures that supported growth. Growth of *P. tinctorius* was better at 30 and 35 C than at other temperatures. This fungus did not grow at 40 C but remained viable. Autolysis was apparent after 60 days' incubation at 25-35 C.

Bioassays of culture filtrates.—Duration of incubation and temperature influenced the production of the antibiotic(s) by *L. cerealis* var. *piceina*. In agar diffusion assays, the nondiluted filtrate from 30- to 60-day

TABLE 4. Mycelial weights (mg) of pine mycorrhizal fungi after 40 days' growth at 25 C in liquid media

Medium	Mycorrhizal fungus					
	Amanita muscaria	*Laccaria laccata*	*Lactarius deliciosus*	*Leucopaxillus cerealis* var. *piceina*	*Pisolithus tinctorius*	*Suillus luteus*
MMN[a]	27.3	31.6	49.0	54.4	66.3	22.4
Hagem	10.2	23.1	16.3	42.9	52.4	21.2

[a] Modified Melin-Norkrans liquid medium.

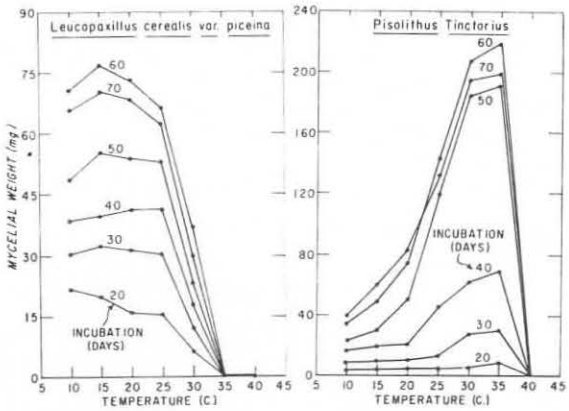

Fig. 2. Growth of *Leucopaxillus cerealis* var. *piceina* and *Pisolithus tinctorius* in MMN liquid medium after different periods of incubation at various temperatures.

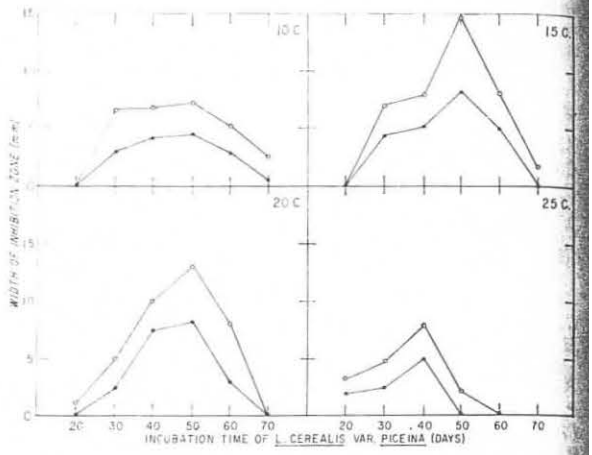

Fig. 3. Influence of culture filtrates of *Leucopaxillus cerealis* var. *piceina* grown in MMN liquid medium at different temperatures for different periods of time in agar-diffusion plates on growth of *Phytophthora cinnamomi* in 5 days at 23 C from zoospore (○——○) and mycelial disc (●——●) inocula.

cultures (incubated at 10 to 20 C) (Fig. 3) inhibited growth of *P. cinnamomi* from zoospore and mycelial disc inocula. Filtrates from cultures grown for 50 days or longer at 25 C did not inhibit growth. Cultures grown for 50 days at 15 and 20 C gave the strongest inhibition. Of the 1:1 (filtrate:control liquid) dilutions, the 50-day, 15-C filtrate was the only one which inhibited growth of *P. cinnamomi*. The antifungal principle in filtrates of *L. cerealis* var. *piceina* inhibited the growth of zoospores of *P. cinnamomi* more than the growth of the fungus from mycelial discs. The controls and *P. tinctorius* filtrate dilutions did not inhibit growth of *P. cinnamomi*, nor did filtrates of *L. cerealis* var. *piceina* from the 30 to 40 C series.

Growth of *P. cinnamomi* directly in culture filtrates was inhibited strongly by 30- to 60-day filtrates of

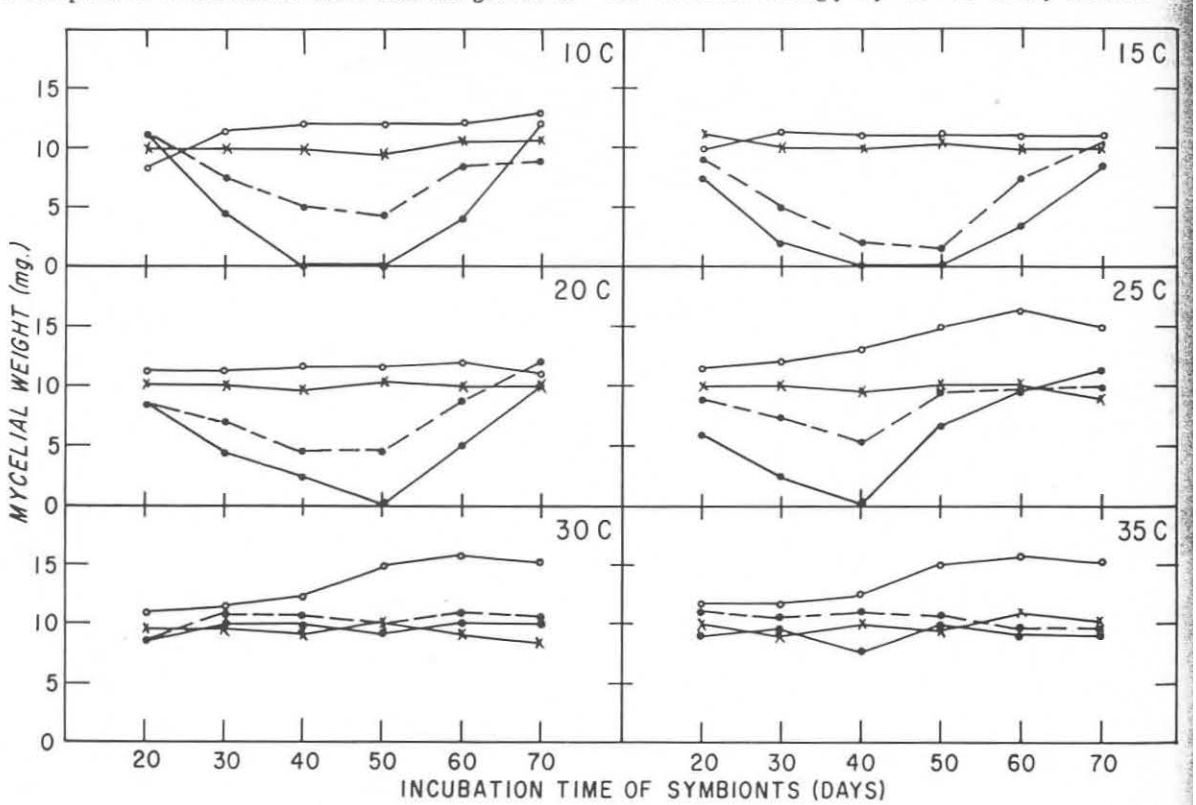

Fig. 4. Growth of *Phytophthora cinnamomi* in 7 days at 23 C in control medium (x——x, nondiluted), and filtrates of *Leucopaxillus cerealis* var. *piceina* (●——●, nondiluted; ●– – –●, 1:2 dilution with control medium) and *Pisolithus tinctorius* (○——○, nondiluted) grown in MMN liquid medium at different temperatures for different periods of time.

L. cerealis var. *piceina* grown at 10 and 20 C (Fig. 4). Only weak antifungal activity was detected in 50-day or older cultures grown at 25 C. No activity was detected in filtrates of *L. cerealis* var. *piceina* from the 30-40 C regimes at any incubation period. The inhibitory principle in filtrates of *L. cerealis* var. *piceina* was fungistatic to *P. cinnamomi* because growth on V-8 agar medium was excellent from mycelial discs previously incubated for 7 days in filtrates which completely inhibited growth; i.e., 40- and 50-day incubation series from 10-15 C and 50-day incubation series from 20 and 25 C. The strongest inhibitory effect was apparent in cultures of *L. cerealis* var. *piceina* grown for 50 days at 15 C. Filtrates of *P. tinctorius* stimulated growth of *P. cinnamomi,* especially that from the older cultures grown at 25-35 C. Correlated with growth stimulation was the presence of increasing amounts of a dark red-brown pigment in filtrates of *P. tinctorius* with increasing incubation time at these temperatures. This pigment was light and pH sensitive; it gradually turned dark red upon exposure to light or, in darkness, after reaction adjustment to pH 6.0. Growth of *P. cinnamomi* was not inhibited in any filtrate from the 40 C series.

Motility and germination of zoospores of *P. cinnamomi* were inhibited strongly in the filtrates of *L. cerealis* var. *piceina* from 40- and 50-day cultures grown at 10, 15, and 20 C. Filtrates from cultures grown for 40 days at 25 C were strongly inhibitory, but filtrates from 50-day cultures at this temperature were only weakly inhibitory. The greatest inhibition took place in the 50-day filtrates of *L. cerealis* var. *piceina* from the 15 and 20 C series, which agrees with the previous assays (Fig. 5). Filtrates from *P. tinctorius* cultures and the control series did not inhibit zoospores, since motility was maintained for 6 to 8 hr, and germination after encystment was between 90 to 100%.

Antibacterial activity was strong, especially in filtrates of *L. cerealis* var. *piceina* from cultures grown for 30 to 60 days at 10 to 25 C (Fig. 6). Potent filtrates of this symbiont were completely bacteriostatic, since only the number of bacteria originally introduced (0.083 million of cells/ml) were recovered after 48 hr incubation. Bacteria were either much more sensitive to the inhibitor(s) produced by *L. cerealis* var. *piceina* than *P. cinnamomi,* or an antibacterial substance was produced in addition to the antifungal principle, which was the first to appear and the last to disappear in culture. *Pisolithus tinctorius* increased bacterial multiplication in the 30-day and older cultures grown at 25 to 35 C. These same filtrate treatments stimulated *P. cinnamomi* in the earlier bioassay.

Discussion.—Certain shortleaf and loblolly pine mycorrhizal fungi produce antibiotics in pure culture. The high percentage (44%) of different root pathogens inhibited by these symbiotic fungi suggests that they may be an important source of new and different antibiotics for possible use in agriculture and medicine.

The ecological significance of antibiotic production

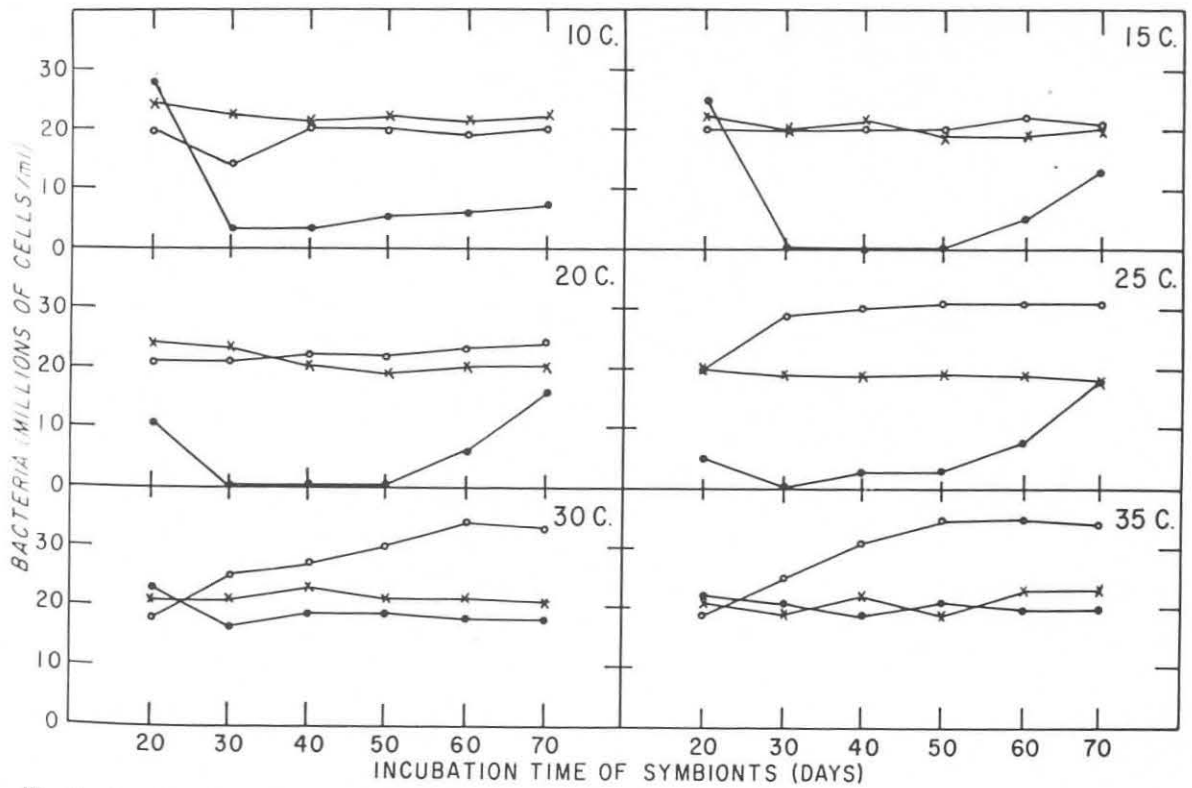

Fig. 5. Number of bacteria after 48 hr incubation at 23 C in control medium (x——x), and in filtrates of *Leucopaxillus cerealis* var. *piceina* (●——●) and *Pisolithus tinctorius* (○——○) grown in MMN liquid medium at different temperatures for different periods of time.

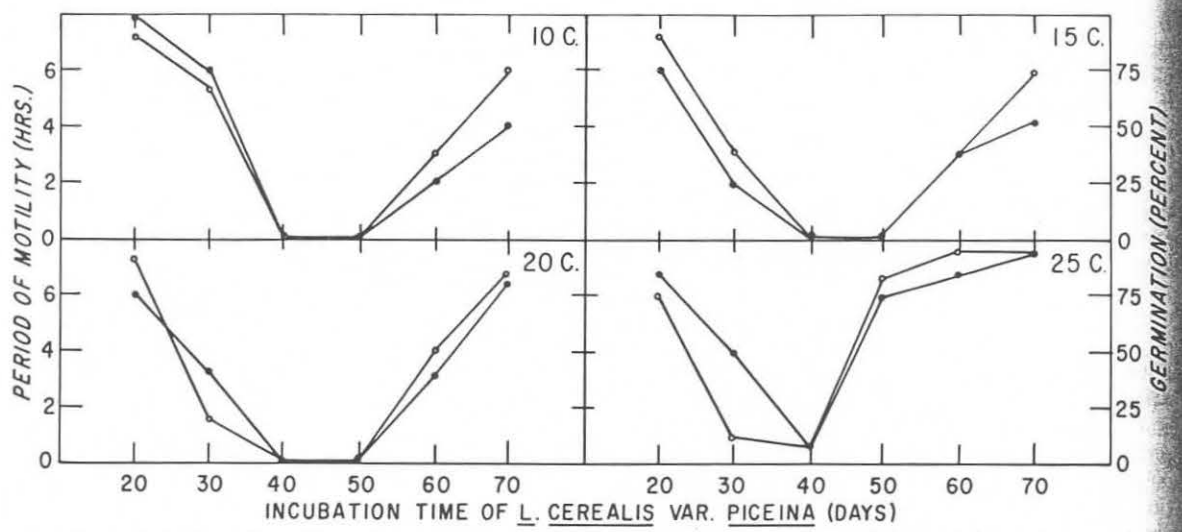

Fig. 6. Period of motility (○——○) and % germination (●——●) of zoospores of *Phytophthora cinnamomi* after 8 hr at 23 C in filtrates of *Leucopaxillus cerealis* var. *piceina* grown in MMN liquid medium at different temperatures for different periods of time.

by these mycorrhizal fungi needs further elaboration. Future research with mycorrhizal fungi, particularly *Leucopaxillus cerealis* var. *piceina,* should include investigations on the influence of antibiotics on growth, survival, and infectivity of root pathogenic fungi.

Maximum production of an antibiotic(s) by *L. cerealis* var. *piceina* during the rapid phase of growth, and the subsequent rapid disappearance of antibiotic activity during the autolytic phase of culture growth, has interesting implications. In past work on production of antibiotics and other waste products by fungi (22), synthesis of these substances was found in most instances to culminate after the rapid phase of growth. The antibiotic(s) produced by *L. cerealis* var. *piceina* may not be a waste product, but rather an essential metabolite with a vital function in the physiology of the fungus. This may be beneficial to the root protection concept because the antibiotic should be produced by the symbiont during early mycorrhizal function, and not just during the gradual dying of these structures. Production of the antibiotic in early mycorrhizal development should insure a more significant inhibitory effect on root pathogens.

The wide extremes of temperature optima for growth of *L. cerealis* var. *piceina* (between 10 and 20 C) and *P. tinctorius* (between 30 and 35 C) are unusual because most other mycorrhizal fungi grow best between 18 and 27 C (7). However, *Suillus ploraus* (Roll.) Sing. has been reported to grow best between 15 and 18 C, and *Lyophyllum immundum* (Berk.) Kuhn (syn. *Tricholoma fumosum*) between 27 and 30 C (8).

Subsequent reports in this series will examine the temperature requirements of *Leucopaxillus cerealis* var. *piceina* and *Pisolithus tinctorius* for mycorrhizal synthesis with pine, the identification of the antibiotics produced by *L. cerealis* var. *piceina,* and their involvement in protection against *P. cinnamomi* infection on roots of pine seedlings.

LITERATURE CITED

1. Bryan, W. C., and B. Zak. 1961. Synthetic cultures of mycorrhizae of southern pines. Forest Sci. 7:123-129.
2. Bryan, W. C., and B. Zak. 1962. Additional syntheses of mycorrhizae on shortleaf and loblolly pines. Forest Sci. 8:1.
3. Cameron, H. R. 1962. Effect of hydrogen ion concentration on growth rates of *Phytophthora* spp. Phytopathology 52:727. (Abstr.)
4. Eglite, A. K. 1958. Trudnorastvorimye mineraly i organicheskie veshchestva v kachestve istochnika pitaniya mikoriznykh gribov. (Slightly soluble minerals and organic substances as nutrients of mycorrhizal fungi.) Akad. Nauk Latv. SSR. Trudy Instit. Mikrobiol. 7:67-75.
5. Gaümann, E. 1960. New data on the chemical defence reactions of orchids. Acad. Sci. (Paris) 250:1944-1947.
6. Gaümann, E., J. Nuesch, and R. H. Rimpau. 1960. Further studies on the chemical defensive reaction in orchids. Phytopathol. Z. 38:274-308.
7. Hacskaylo, E., J. G. Palmer, and J. A. Vozzo. 1965. Effect of temperature on growth and respiration of ectotrophic mycorrhizal fungi. Mycologia 57:748-756.
8. Harley, J. L. 1959. The biology of mycorrhiza. Interscience Publishers, Inc., New York. 234 p.
9. Harley, J. L., and J. M. Wilson. 1959. Absorption of potassium by beech mycorrhiza. New Phytol. 58: 281-298.
10. Hatch, A. B. 1937. The physical basis of mycotrophy in *Pinus*. Black Rock Forest Bull. 6. 168 p.
11. Landy, M., S. B. Rosenman, and G. H. Warren. 1947. An antibiotic from *Bacillus subtilis* active against pathogenic fungi. J. Bacteriol. 54:24.
12. Marx, D. H., and F. A. Haasis. 1965. Induction of aseptic sporangial formation in *Phytophthora cinnamomi* by metabolic diffusates of soil micro-organisms. Nature 206:673-674.
13. Melin, E. 1952. Transfer of labelled nitrogen from an ammonium source to pine seedlings by means of mycorrhizal hyphae. Physiol. Plantarum 3:88-92.
14. Melin, E. 1955. Ca^{45} used as indicator of transport of cations to pine seedlings by means of mycorrhizal mycelium. Svensk Botan. Tidskr. 49:119-122.
15. Melin, E., H. Nilsson, and E. Hacskaylo. 1958.

Translocation of cations to seedlings of *Pinus virginiana* through mycorrhizal mycelium. Botan. Gaz. 199:243-246.

16. MODESS, O. 1941. Zur Kenntnis der Mykorrhizabildner von Kiefer und Fichte. Symb. Bot. Upsal. 5:1-147.
17. MORRISON, T. M. 1962. Absorption of phosphorus from soils by mycorrhizal plants. New Phytol. 61: 10-20.
18. NORKRANS, B. 1949. Some mycorrhiza-forming *Tricholoma* species. Svensk Botan. Tidskr. 43:485-490.
19. ROSENDAHL, R. O. 1942. The effect of mycorrhizal and non-mycorrhizal fungi on the availability of difficultly soluble potassium and phosphorus. Soil Sci. Soc. Amer. Proc. 7:477-479.
20. ROUTIEN, J. B., and R. F. DAWSON. 1943. Some interrelationships of growth, salt absorption, respiration and mycorrhizal development in *Pinus echinata*. Am. J. Botany 30:440-449.
21. SINGER, R. 1962. The Agaricales in modern taxonomy. (2nd ed.), J. Cramer, Weinheim. 915 p.
22. VANÉK, Z., and Z. HOŠTALEK. 1965. Biogenesis of antibiotic substances. Academic Press, New York. 324 p.
23. ZAK, B. 1964. Role of mycorrhizae in root disease. Annu. Rev. Phytopathol. 2:377-392.